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(54) Title: KINASE BLOCKING POLYPEPTIDES AND USES THEREOF

(57) Abstract: Disclosed are peptides (blocking polypeptides) that inhibit kinase Eg2 activity, thereby inhibiting activation of the protein known as CPEB. DNAs encoding the CPEB-polypeptide, vectors, and cells containing the DNA, and CPEB-like specific antibodies are also disclosed.

KINASE BLOCKING POLYPEPTIDES AND USES THEREOF

Field of the Invention

This invention relates to intracellular signaling and methods to control intracellular signaling pathways.

Background of the Invention

Following transcription in the nucleus, many mRNAs are exported to the cytoplasm and are stored in a translationally dormant state. Their timed expression is important for various processes including early animal development. For example, in Xenopus oocytes dormant mRNAs are activated during oocyte maturation causing cells to re-enter meiosis. These dormant mRNAs encode a variety of developmentally important products including those that drive the cell cycle, establish polarity and determine cell fate. Examples of proteins specifically translated from dormant mRNAs include Mos, cyclins and cdk2.

A mechanism that controls the translation of dormant mRNA is cytoplasmic poly (A) elongation. Cytoplasmic adenylation requires two cis elements in the 3'-untranslated region (UTR) of a responding mRNA. The two elements are (a) the hexanucleotide AAUAAA, and (b) the cytoplasmic polyadenylation element (CPE), which usually resides within 50 bases upstream of the hexanucleotide. An RNA binding protein, CPEB, modulates polyadenylation by binding to CPE and effecting the timing and extent of translational activation (Hake et al., *Biochim. Biophys. Acta,* 1332, M31-M38 (1997) and Stebbins-Boaz et al. *Crit. Rev. Eukaryot. Gene Expr.,* 7:73-94 (1997)).

In mammals, CPEB is present in oocytes, tissues involved in the immune response, and in the brain, particularly in the dendritic layer of the hippocampus and at synapses of cultured hippocampal neurons (Wu et al, *Neuron*, 21:1129-1139, 1998). In response to synaptic stimulation, a CPE-containing mRNA encoding a-calmodulin-dependent protein kinase II undergoes polyadenylation and translational activation.

Summary of the Invention

The invention is based on the discovery that Xenopus CPEB is phosphorylated by the serine\threonine kinase Eg2, and that the phosphorylation occurs at serine residue 174, found in a conserved motif consisting of amino acids LDSR. Moreover, an ovalbumin-linked polypeptide corresponding to amino acid residues 163-181 of the RNA binding protein CPEB competitively inhibits CPEB phosphorylation *in vivo* and delays progesterone-induced maturation of Xenopus oocytes. In addition, polypeptides which contain the LDXR motif have been found to be useful for inhibiting Eg2 activity. Since Eg2 functions as a kinase and plays a role in intracellular signaling, the present invention provides polypeptides which can be used to control Eg2 activity. For example, Eg2 is involved in cancerous cell growth. Thus, the polypeptides of the invention can be used to treat cancer.

Based on these discoveries, the invention features a substantially pure polypeptide of 5-100 amino acids ("blocking polypeptide"), which includes the amino acid sequence LDXR (SEQ ID NO: 15), wherein "X" is G, A, V, L, I, M, C, S or T. X can be A, V, L, I, S or T. When X is S or T, it can be phosphorylated or non-phosphorylated, depending on the blocking polypeptide's intended use. The blocking polypeptide can contain about 5 to 100 amino acids, e.g., about 5, 6, 7, 8, 9, 10-20, 30, 40, 50, 60, 70, 80, or 90 amino acid residues. Suitable blocking polypeptides include fragments or variants of CPEB from various organisms, e.g., Xenopus CPEB (LRSSRLDSRSILDSRSS; SEQ ID NO:3) or mouse CPEB (RGSRLDTRPILDSR; SEQ ID NO:4). The CPEB polypeptides can be phosphorylated, e.g., the serine at position 8 of the Xenopus sequence LRSSRLDSRSILDSRSS (SEQ ID NO:3) can be phosphorylated; or the threonine at position 7 of the mouse sequence RGSRLDTRPILDSR (SEQ ID NO:4) can be phosphorylated. Exemplary blocking polypeptides also include LRSSRLDXRSILDSRSS (SEQ ID NO:5) or RGSRLDXRPILDSR (SEQ ID NO:6), with one or more conservative amino acid substitutions therein, provided that the LDXR motif is preserved. Specific embodiments, of the blocking polypeptides are LRSSRLDARSILDSRSS (SEQ ID NO:7) and RGSRLDARPILDSR (SEQ ID NO:8).

Also within the invention are isolated nucleic acid sequences that encode the blocking polypeptide described above.

The invention also includes a vector containing the above-described nucleic acid sequence, and a cell containing the vector. The cell can be prokaryotic or eukaryotic, e.g., an animal cell such as a mammalian cell.

In another aspect, the invention includes a method of producing a blocking polypeptide. The method includes culturing a cell containing a nucleic acid sequence which encodes a blocking polypeptide described herein under conditions and for a time sufficient to enable the cell to express a polypeptide encoded by the nucleic acid, and isolating the blocking polypeptide from the cell.

Antibodies specific for the blocking polypeptides described herein are also within the invention. The antibody can be polyclonal or monoclonal. In one embodiment, the blocking polypeptide is phosphorylated, e.g., at amino acid X of the LDXR motif and the antibody is specific for the phosphorylated form of the blocking polypeptide. In another embodiment, the antibody can be bound to a solid support.

The invention also features a method (e.g., in vivo or in vitro) of inhibiting the activity of an Eg2 including contacting Eg2 with an effective amount of a blocking polypeptide described herein.

Also within the invention is a method of inhibiting CPEB phosphorylation in a cell by contacting Eg2 in the cell with an effective amount of a blocking polypeptide described herein. The method can be performed *in vivo*, *ex vivo*, or *in vitro*.

Other methods, such as a method of detecting phosphorylated CPEB, e.g., phosphorylated CPEB, are also within the invention. The method includes contacting an antibody, that specifically binds to a blocking polypeptide described herein, to a sample, e.g., a cell, under conditions that enable the antibody to bind to phosphorylated CPEB to form a CPEB-antibody complex, if present, and detecting any complexes, the presence of a complex indicating the presence of CPEB, or phosphorylated CPEB, in the sample. The method can be performed in vivo or in vitro.

The invention also includes a method of treating a cancer cell. The method includes contacting a cell with an effective amount of blocking polypeptide described herein, thereby treating the cancer cell. This method can be performed *in vivo* or *in vitro*.

An "isolated DNA" is a DNA free of the genes that flank the DNA in the genome of the organism in which the DNA naturally occurs. The term therefore includes a recombinant DNA incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote. It also includes a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment.

A gene and a regulatory sequence(s) are "operably linked" when they are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

At "substantially pure polypeptide" is a polypeptide separated from components with which it is naturally associated. A polypeptide is substantially pure when it is at least 60% by weight, free from the proteins and other naturally-occurring organic molecules with which it is naturally associated. For example, the purity of the preparation is at least 75%, at least 90%, or at least 99%, by weight. A substantially pure polypeptide can be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding a polypeptide, or by chemical synthesis. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. A chemically synthesized polypeptide or a recombinant polypeptide produced in a cell type other than the cell type in which it naturally occurs is, by definition, substantially free from components that naturally accompany it. Accordingly, substantially pure polypeptides include those having sequences derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

A "vector" is a replicable nucleic acid construct. Examples of vectors include plasmids and viral nucleic acids.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the

present document, including definitions, will control. Unless otherwise indicated, materials, methods, and examples described herein are illustrative only and not intended to be limiting.

Various features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Drawings

Fig. 1 is a representation of a sequence comparison between Xenopus (SEQ ID NO:12), mouse (SEQ ID NO:13), and zebrafish CPEBs (SEQ ID NO:14). Identical or similar amino acids are boxed. The phosphoserine is identified by a star.

Figs. 2A and 2B are histograms showing that CPEB is required for oocyte maturation. Fig. 2A and 2B show % germinal vesicle break-down (GVBD, marker of oocyte maturation) of oocytes injected with (i) wild type CPEB (WT; column 3 and column 4 of Fig. 2A and column 9 or 10 of Fig. 2B), (ii) alanine CPEB varient (AA; column 5 and 6 of Fig. 2A) and (iii) aspartic CPEB varient (DD; column 11 and 12 of Fig. 2B) in the presence of progesterone (column 4 or 6 of Fig. 2A and column 10 or 12 of Fig. 2B) or absence of progesterone (column 3 or 5 of Fig. 2A and column 9 or 11 of Fig. 2B). The % GVBD in control oocytes is shown in column 1 and 2 of Fig. 2A and column 7 and 8 of Fig. 2B.

Fig. 3 is a line graph showing % GVBD of oocytes injected with the ovalbumin-linked blocking polypeptide at 1, 2, 3, 4, or 5 hours in the presence and absence of progesterone. BSA was injected into oocytes as a control.

Detailed Description

The invention encompasses polypeptides (referred to herein as "blocking polypeptides") that can inhibit the activity of Eg2. Eg2 is a member of the Aurora family of protein kinases and is known to play a role in cell signaling. For example, Eg2 plays a role in early animal development and embryogenesis and, in the brain, can mediate synaptic plasticity. Accordingly, the blocking polypeptides of the invention can be used to inhibit Eg2 activity, and control Eg2 activity. In another example, Eg2 is involved in cancerous cell growth. Inhibition of Eg2 activity using the polypeptides of the invention can be used to treat cancer by inhibiting Eg2 activity.

Polypeptides

The invention features blocking polypeptides between 6-100 amino acids in length, and contains the amino acid sequence LDXR (SEQ ID NO: 7), wherein "X" is G, A, V, L, I, M, C, S, or T. Exemplary blocking polypeptides are fragments or variants of CPEB. The CPEB fragments or variants can be from any of various organisms such as Xenopus, mouse, human, rat, pig, horse, cow or rabbit. Suitable blocking polypeptides include LRSSRLDXRSILDSRSS (SEQ ID NO:5) or RGSRLDXRPILDSR (SEQ ID NO:6), or those same peptides with one or more conservative amino acid substitutions therein. The replacement of an amino acid of a blocking polypeptide with a conservative amino acid preferably results in a polypeptide which retains the function of the original blocking polypeptide, e.g., inhibits Eg2 activity. "Conservative" amino acid substitutions are substitutions in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Any one of a family of amino acids can be used to replace any other members of the family in a conservative substitution.

The blocking polypeptides can be used, e.g., to inhibit Eg2 activity, inhibit phosphorylation of CPEB, or to distinguish between phosphorylated and non-phosphorylated CPEB in an organism, e.g., an animal, e.g., a human. It has been discovered that a blocking polypeptide with the X position occupied by an alanine residue is particularly effective for inhibiting Eg2 activity. Without intending to be bound by theory, the inventors observe that this may result from the A-substituted blocking polypeptide remaining bound in the Eg2 active site.

A blocking polypeptide can be isolated and purified from a natural source. Alternatively, it can be chemically synthesized by conventional methods. Methods for synthesizing polypeptides include solid phase synthesis as described by Merdfield (*J. Am. Chem. Soc.* 85:2149 (1963)), fragment condensation or classical solution synthesis. Automated synthesizers such as the Beckman, Applied Biosystem Inc. (Rao et al., *Int. J, Pep. Prot. Res.* 40:508-515 (1992), Vega 250 automated peptide synthesizer, or other peptide synthesizers can be employed to synthesize the polypeptides of the present invention.

Recombinant DNA methodology can also be used to prepare the blocking polypeptides. A typical method involves transfecting host cells (*e.g.*, bacterial cells, human cells, or Xenopus cells) with an expression vector containing a nucleotide sequence that encodes a blocking polypeptide. The recombinant blocking polypeptide can be purified from the culture medium or from lysates of the cells.

A phosphorylated form of the blocking polypeptide can be generated by methods known in the art. For example, the blocking polypeptide can be phosphorylated by incubating the blocking polypeptide with a Eg2 source, e.g., progesterone-stimulated oocyte cell extracts or with recombinant Eg2. Alternatively, a phosphorylated blocking polypeptide can be made chemically, using known methods that are commercially available.

Also within the invention are fusion proteins or polypeptides that include the blocking polypeptides of the invention fused to an unrelated protein. The unrelated protein can be selected to facilitate purification, detection, solubilization, or to provide some other function. Fusion proteins can be produced synthetically or the blocking polypeptide can be linked to an unrelated protein using an appropriate coupling reagent, e.g., dicyclohexylcarbodiimide (DCC). Alternatively, fusion proteins can be produced recombinately by cloning a nucleotide sequence that expresses the fusion protein into an appropriate expression vector. The recombinant fusion polypeptide can then be purified from the culture medium or from lysates of the cells.

The blocking polypeptides can be purified using HPLC, gel filtration, ion exchange chromatography, or other known methods.

The "blocking polypeptides" of this invention can be used to treat cancer, inhibit synaptic function, or inhibit oocyte maturation or embryogenesis

DNA Molecules and Vectors

The invention includes an isolated DNA molecule that encodes a blocking polypeptide described above. Those of skill in the art will recognize that because of the degeneracy of the genetic code, numerous different nucleotide sequences can be used to encode any given blocking polypeptide. However, codons can be chosen for optimal expression in the host organism. Isolated DNAs for use in the invention include DNA fragments or variants of wild type CPEB that contain a sequence that encodes an LDXR motif. The CPEB DNA for use in the present invention can be from any of various organisms, including Xenopus, mouse, human, rat, pig, horse, cow and rabbit. Preferred DNA sequences of the present invention include Xenopus CPEB 5'-tgcgtagctctcgattggacagccgctctattttggattctcgctccagc- 3' (SEQ ID NO:1) or mouse CPEB 5'-agaggatctcgcctggacacccggcccatcctggactcccgt-3' (SEQ ID NO:2). The complete DNA sequence of mouse (NIH accession number Y08260) and Xenopus CPEB (NIH accession number U14169) are known in the art.

The DNA of this invention can be used to treat cancer, inhibit synaptic function, or control oocyte maturation or embryogenesis. Alternatively, it can be used to produce a recombinant blocking polypeptide. For such uses, the DNA of the present invention is typically cloned into an expression vector, i.e., a vector wherein DNA is operably linked to expression control sequences. The need for, and identity of, expression control sequences will vary according to the type of cell in which the DNA is to be expressed. Generally, expression control sequences include a transcriptional promoter, enhancer, suitable mRNA ribosomal binding sites, and sequences that terminate transcription and translation. Suitable expression control sequences can be selected by one of ordinary skill in the art.

Conventional methods can be used by the skilled person to construct expression vectors. *See generally*, Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual* (2nd Edition), Cold Spring Harbor Press, N.Y. Vectors useful in this invention include plasmid vectors and viral vectors. Preferred viral vectors are those derived from retroviruses, adenovirus, adeno-associated virus, SV40 virus, or herpes viruses.

In some embodiments of the invention, the DNA is introduced into, and expressed in, a prokaryotic cell. A preferred prokaryotic cell is *Escherichia coli*. For expression in a prokaryotic cell, the DNA can be integrated into a bacterial chromosome or expressed from an extrachromosomal DNA.

In other embodiments of the invention, the DNA is introduced into, and expressed in, a eukaryotic cell *in vitro*. Eukaryotic cells useful for expressing DNA *in vitro* include, but are not limited to, COS, CHO, and Sf9 cells. Transfection of the eukaryotic cell can be transient or stable. The DNA can be, but is not necessarily, integrated into a chromosome of the eukaryotic cell.

Specific Antibodies

Antibodies specific for the blocking polypeptide can be raised by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with an immunogenic preparation that contains the blocking polypeptide. An appropriate immunogenic preparation can contain, for example, a recombinantly expressed blocking polypeptide or a chemically synthesized blocking polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic blocking polypeptide preparation induces a polyclonal anti-blocking polypeptide antibody response. Antibodies specific for the phosphorylated form of the blocking polypeptide can also be generated and used, e.g., to determine if phosphorylated CPEB is present in a cell. The term antibody refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules. Examples of immunologically active portions of immunoglobulin molecules include F (ab) and F $(ab')_2$ fragments, which can be generated by treating the antibody with an enzyme such as pepsin. The term monoclonal antibody or monoclonal antibody composition refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of a polypeptide. A monoclonal antibody composition thus typically displays a single binding affinity for the blocking polypeptide with which it immunoreacts.

Polyclonal anti-blocking polypeptide antibodies can be prepared by immunizing a suitable subject with a polypeptide immunogen. The anti-blocking polypeptide antibody titer in the immunized subject can be monitored over time by well-known techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired, the antibody molecules directed against the polypeptide can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as protein A chromatography, to obtain the IgG fraction. Anti-blocking polypeptide antibodies against the phosphorylated form of the blocking polypeptide can also be generated using methods known in the art. For example, the phosphorylated form of the protein can be immunized into a rabbit. Following isolation of antibodies, the IgG fraction is purified with protein A-agarose. Phosphorylated peptide-specific IgG antibodies are then purified by first passing the IgG over immobilized, nonphosphorylated blocking polypeptides to remove antibodies reactive with nonphophorylated epitopes. The nonadsorbed fraction is then passed over a column of immobilized phosphorylated blocking polypeptides. After extensive washing, the retained immunoglobulins are eluted at low pH, rapidly neutralized, dialyzed and concentrated.

Monoclonal antibodies can be generated by immunizing a subject with an immunogenic preparation containing a blocking polypeptide. At an appropriate time after immunization, e.g., when the anti-polypeptide antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by techniques well known in the art, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497, the human B cell hybridoma technique (Kozbor et al. (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96), or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (*see generally* Current Protocols in Immunology (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New York, NY). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with a blocking polypeptide immunogen as described above, and the culture supernatant of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds to the blocking polypeptide.

In addition to preparing monoclonal antibody-secreting hybridomas, one can identify and isolate monoclonal anti-blocking polypeptide antibodies by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with the blocking polypeptide to thereby isolate immunoglobulin library members that bind to the blocking polypeptide. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display libraries can be found in, for example, U.S. Patent No. 5,223,409; PCT Publication No. WO 92/18619; and PCT Publication No. WO 91/17271.

Additionally, recombinant anti-blocking polypeptide antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT Publication No. WO 87/02671; European Patent Application 184,187; European Patent Application 171,496; and European Patent Application 173,494.

An anti-polypeptide antibody (e.g., monoclonal antibody) can be used to isolate a polypeptide using techniques well known in the art, such as affinity chromatography or immunoprecipitation. An anti-blocking polypeptide antibody can facilitate the purification of recombinantly produced polypeptide expressed in host cells. Moreover, an anti-blocking polypeptide antibody can be used to detect a blocking polypeptide (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the polypeptide. Anti-blocking polypeptide antibodies can be used diagnostically, e.g., to detect CPEB or phosphorylated CPEB in a cell, e.g., a neuron. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, α-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and

avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H.

Uses of antibodies include inhibiting the activity of endogenous CPEB, detecting CPEB polypeptides in immunohistochemical methods and immunoassays, and purifying CPEB polypeptides. Antibodies generated against the phosphorylated form of the CPEB polypeptide can be used to distinguish between phosphorylated and non-phosphorylated CPEB; to identify cells in which CPEB is phosphorylated; and to identify cells in which Eg2 is active. Since Eg2 has a role in the brain mediating synaptic plasticity, antibodies against Eg2 may be useful for inhibiting neural development, learning and memory. By administering these antibodies to an animal, one can generate an animal model for studying Alzheimers disease. Alternatively, Eg2 can contribute to the over-proliferative activity of cells, e.g., cancer. Thus, the antibodies can be useful to treat cancer.

The antibodies of the present invention can be bound to a solid support, e.g., polystyrene beads, cross-linked beaded agaroses, or Protein A-Sepharose CL-4B (Sigma).

Pharmaceutical Formulation and Administration

When administered to an animal or a human, e.g., to treat cancer, modulate synaptic function, or inhibit oocyte maturation, the new DNAs, blocking polypeptides, or antibodies can be used alone, or in a mixture, in the presence of a pharmaceutically acceptable excipient or carrier (e.g., physiological saline). The excipient or carrier is selected on the basis of the mode and route of administration, and well known pharmaceutical practice. Suitable pharmaceutical carriers, as well as pharmaceutical necessities for use in pharmaceutical formulations, are described in *Remington's Pharmaceutical Sciences* (E. W. Martin), a well known reference text in this field, and in the USP/NF.

Alternatively, the therapeutic compositions can be formulated to include ingredients that augment or potentiate the therapeutic activity of the blocking polypeptides, e.g., those that increase the biological stability of the polypeptides.

Preferably, the therapeutic compositions of the invention are administered locally to a target tissue or cell. For example, the blocking polypeptides or antibodies of the invention can be directly injected into a tumor. Administration of a therapeutic composition may be repeated as needed, as determined by one skilled in the art.

Treatment includes administering a pharmaceutically effective amount of a composition containing a blocking polypeptide to a subject in need of such treatment, thereby inhibiting or reducing cancer cell growth in the subject. Such a composition typically contains from about 0.1 to 90% by weight (such as 1 to 20% or 1 to 10%) of a blocking polypeptide in a pharmaceutically acceptable carrier.

Solid formulations of the compositions for oral administration can contain suitable carriers or excipients, such as corn starch, gelatin, lactose, acacia, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, calcium carbonate, sodium chloride, or alginic acid. Disintegrators that can be used include, without limitation, micro-crystalline cellulose, corn starch, sodium starch glycolate and alginic acid. Tablet binders that can be used include acacia, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone (Povidone®), hydroxypropyl methylcellulose, sucrose, starch, and ethylcellulose. Lubricants that can be used include magnesium stearates, stearic acid, silicone fluid, talc, waxes, oils, and colloidal silica.

Liquid formulations of the compositions for oral administration prepared in water or other aqueous vehicles can contain various suspending agents such as methyl cellulose, alginates, tragacanth, pectin, kelgin, carageenan, acacia, polyvinylpyrrolidone, and polyvinyl alcohol. The liquid formulations can also include solutions, emulsions, syrups and elixirs containing, together with the active compound(s), wetting agents, sweeteners, and coloring and flavoring agents. Various liquid and powder formulations can be prepared by conventional methods for inhalation into the lungs of the mammal to be treated.

Injectable formulations of the compositions can contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol, and the like). For intravenous injections, water soluble versions of the compounds can be administered by the drip method, whereby a pharmaceutical formulation containing the

blocking polypeptide and a physiologically acceptable excipient is infused. Physiologically acceptable excipients can include, for example, 5% dextrose, 0.9% saline, Ringer's solution, or other suitable excipients. For intramuscular preparations, a sterile formulation of a suitable soluble salt form of the compounds can be dissolved and administered in a pharmaceutical excipient such as Water-for-Injection, 0.9% saline, or 5% glucose solution. A suitable insoluble form of the compound can be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, such as an ester of a long chain fatty acid, (e.g., ethyl oleate).

A topical semi-solid ointment formulation typically contains a concentration of the active ingredient from about 1 to 20%, e.g., 5 to 10% in a carrier such as a pharmaceutical cream base. Various formulations for topical use include drops, tinctures, lotions, creams, solutions, and ointments containing the active ingredient and various supports and vehicles.

The optimal percentage of the blocking polypeptides in each pharmaceutical formulation varies according to the formulation itself and the therapeutic effect desired in the specific pathologies and correlated therapeutic regimens. Appropriate dosages of the blocking polypeptides can be determined by those of ordinary skill in the art of medicine by monitoring the subject for signs of disease amelioration or inhibition, and increasing or decreasing the dosage and/or frequency of treatment as desired. The optimal amount of the blocking polypeptides used for treatment of conditions caused by or contributed to by Eg2, e.g., cancer, depends upon the manner of administration, the age and the body weight of the subject, and the condition of the subject to be treated. Generally, the blocking polypeptides compound are administered at a dosage of 0.01 to 100 mg/kg body weight.

Use of Blocking Polypeptides and Antibodies

The blocking polypeptides can be used as a research tool or clinically to inhibit the activity of Eg2. For example, the blocking polypeptides can be used to inhibit early animal development and embryogenesis by inhibiting phosphorylation of CPEB by Eg2 in maturing oocytes. The blocking peptides thus provide the opportunity to situate events in a cell and determine the exact developmental control point for CPEB.

The blocking polypeptides can also be used to inhibit Eg2 phosphorylation of CPEB in the brain. Since CPEB phosphorylation in the brain mediates synaptic plasticity, the blocking polypeptides can be used to inhibit synaptic plasticity and are useful for inhibiting neural development, learning and memory. Administration of the blocking peptides to an animal serves to generate an animal model for studying Alzheimers disease. This animal model can be used to screen for drugs that enhance synaptic plasticity.

Eg2 has been observed to be overexpressed in many human cancers including breast, colon, ovarian, prostate, neuroblastoma, and cervical cancers. See, e.g., Bischoff et al., *EMBO J.* 17:3052-3065 (1998); Zhou et al., *Nat. Genet.* 20:189-193 (1998); Sen et al., *Oncogene* 14:2195-2200 (1997); Tatsuka et al., *Cancer Res.* 58:4811-4816 (1998); Tanaka et al., *Cancer Res* 59:2041-2044 (1999); Kimura et al., *J. Biol. Chem.* 274:7334-7340 (1999) and Katayama et al., *Gene* 224:1-7 (1998). The blocking polypeptides can be used to treat cancer by inhibiting Eg2 activity, e.g., the blocking polypeptides can be administered to a prostate cell suspected of being cancerous. In one embodiment, the blocking polypeptides are linked to a tumor-specific targeting moiety, e.g., an antibody, which can be used to deliver the polypeptides to a selected tumor.

Examples

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Example 1: Detection of early CPEB phosphorylation

To detect an early CPEB phosphorylation, stage VI *Xenopus* oocytes were metabolically labeled with ^{32}P and then stimulated to mature with progesterone. Oocytes were collected at different time points before progesterone, 2 hours after progesterone, and at maturation. After immunoprecipitation, CPEB was resolved by SDS-PAGE and detected by Western blotting, and the phosphorylation state analyzed by autoradiography. CPEB underwent a low level of phosphorylation even in immature oocytes and was heavily phosphorylated early during maturation at a time commensurate with c-mos messenger RNA (mRNA) polyadenylation. This early phosphorylation did not result in an altered mobility of CPEB, which was the case late in maturation. This demonstrated that CPEB underwent multiple, time-dependent phosphorylation events during maturation. The early phosphorylation of CPEB triggers maturation of oocytes.

Example 2: Early CPEB phosphorylation sites

To determine the site(s) of the early CPEB phosphorylation, an *in vitro* assay was developed. Recombinant histidine-tagged CPEB was phosphorylated using extracts from non-stimulated or progesterone-stimulated oocytes as the kinase source. However, to first be certain that exogenous CPEB would be phosphorylated in a manner similar to endogenous CPEB, a 2-dimensional phospho-peptide analysis of the two proteins following ^{32}P labeling and trypsin digestion was performed. Metabolically labeled CPEB was immunoprecipitated from mature oocytes and incubated with ^{32}P. Recombinant His-tagged CPEB was phosphorylated *in vitro* with extracts from mature stage VI *Xenopus* oocytes as the kinase source in the presence of ^{32}P. This CPEB was then purified by nickel-chromatography CPEB (recombinant CPEB). Both endogenous and recombinant CPEBs were resolved by SDS-PAGE, Western-blotted, digested with trypsin, and the resultant phosphopeptides were analyzed by two-dimensional peptide mapping followed by autoradiography.

A single tryptic phospho-peptide (Ppl) was detected when recombinant CPEB was phosphorylated in extracts from oocytes incubated with progesterone for a period of time shorter than that required for $p34^{cdc2}$ activation. However, when extracts from fully mature

oocytes were used to phosphorylate CPEB, eight phospho-peptides were detected, which had two-dimensional migration patterns similar to those observed for endogenous CPEB. The phospho-peptides Pp2-Pp8 correspond to likely $p34^{cdc2}$ phosphorylation sites as indicated by peptide sequencing.

To determine whether CPEB phosphorylation in the translational recruitment of c-mos mRNA preceded Mos synthesis, oocytes were first injected with an antisense oligonucleotide to destroy c-mos mRNA, and then incubated in progesterone-containing medium. This treatment prevented Mos protein accumulation and subsequent $p34^{cdc2}$ kinase activation, as well as the CPEB mobility shift and late polyadenylation events, such as those that take place on cyclin Bl and histone B4 mRNAs. Extracts prepared from these oocytes were still able to phosphorylate Ppl, but not Pp2-Pp8. The data indicated that progesterone induced the phosphorylation of a single CPEB peptide prior to Mos synthesis, and suggested that the modification of Ppl was responsible for activation of CPEB. Therefore, Ppl was extracted from the TLC plate, HPLC-purified, and sequenced. The peptide contained the sequence LDSR (SEQ ID NO:15) (residues 172-175 of CPEB), where serine 174 was the phosphorylated residue. This LDSR, and related LDTR motif, are conserved in all known vertebrate CPEBs, and are tandemly repeated in the *Xenopus* and mouse proteins.

Example 3: Ser174 Phosphorylation

To assess whether Ser174 phosphorylation plays a role in the regulation of CPEB activity, site-directed mutagenesis, in which not only Ser174, but also Ser180, were replaced by alanine or aspartic acid residues, was performed. Although Serl80 phosphorylation was not detected, Ser180 was mutated to prevent a possible cryptic phosphorylation event that could mask the importance of changes of Ser174. The CPEB mutants were constructed as described below. Point mutations were made by using a Chamaleon mutagenesis kit (Stratagene, La Jolla, CA) as instructed by the supplier. The selection primer was located in the pMyc-CPEB 5' -CCTCGAGGGGCGGGCCCGTACCCAATTCGCCC-3' (SEQ ID NO:9) and changed a KpnI site to a SrfI site. This primer was used in conjunction with the following mutation primers to create new clones: Serine 174/180 to alanine substitution (PMyc-AA-CPEB) 5'-GCTCTCGATTGGACGCCCGGTCTATTTTGGATGCTCGCTCC-3' (SEQ ID NO:10), and Serine 174/180 to aspartic acid substitution (pMyc-DD-CPEB) 5'-GCTCTCGATT GGACGATCGCTCTATTTTGGATGATCGCTCC-3'(SEQ ID NO:11). The Histagged form of the mutant CPEBs was obtained by subclonning the NcoI-BamHI fragment of Pmyc-AA-CPEB and Pmyc-DD-CPEB in to the NcoI-and BarnHI sites of pHis-CPEB (Stebbins-Boaz et al. *EMBO J* 15:2582-2592 (1997).

Messenger RNA encoding CPEB with the Ser174Ala and Serl80Ala was injected into oocytes, which were also injected with a c-mos 3'UTR fragment to examine polyadenylation. While mRNA encoding wild type CPEB had no effect on progesterone-induced c-mos RNA polyadenylation, the mRNA encoding CPEB with the two alanine substitutions (AA) completely prevented the polyadenylation of this RNA. Moreover, the injection of this mutant CPEB mRNA also prevented endogenous Mos synthesis, as well as oocyte maturation. The data demonstrated that the activation of CPEB requires Ser174 phosphorylation.

While the alanine substitutions caused CPEB to act in a dominant negative fashion, the effect of permanent negative charges in the 174 and 180 positions caused CPEB to act as a dominant gain-of-function mutant. Serl74Asp and Serl80Asp CPEB mutations were constructed, and mRNA encoding this protein was injected into oocytes. In oocytes incubated in the absence of progesterone, mRNA encoding wild type CPEB did not induce

the polyadenylation of c-mos RNA. However, mRNA encoding CPEB with the two Asp substitutions (DD) stimulated the polyadenylation of this RNA in the absence of progesterone. While this polyadenylation was not as robust as that observed in oocytes incubated with progesterone, it was sufficient to induce detectable levels of endogenous Mos accumulation and a very high incidence of oocyte maturation. The data demonstrated that CPEB phosphorylation was sufficient to trigger cytoplasmic polyadenylation and subsequent signaling cascades that result in meiotic progression.

Example 4: Role of Kinase Eg2

Whether Eg2 could be responsible for the phosphorylation of CPEB Ser174 was determined. Baculovirus-expressed Eg2 (NIH accession number Z17206) phosphorylated the same tryptic peptide of recombinant CPEB *in vitro* (Ppl) as was phosphorylated *in vivo* during progesterone-stimulated oocyte maturation. Moreover, the Ser174Ala and Serl80Ala mutant CPEB, which was not phosphorylated in egg extracts, was also not phosphorylated by Eg2, suggesting that the regulatory Ser174 residue was a target of this kinase. In addition, an ovalbumin-linked peptide corresponding to amino acids 163-181 of CPEB of SEQ ID NO:2, which included the motif present in Ppl (ova-pep), was phosphorylated *in vitro* using egg extract or purified recombinant Eg2. This peptide was also able to compete for the phosphorylation of CPEB by Eg2. Finally, the injection of this ovalbumin-linked peptide into oocytes delayed progesterone-induced maturation, as would be expected of a competitive inhibitor of CPEB phosphorylation. The data indicated that Eg2 is the regulatory kinase responsible for the phosphorylation and activation of CPEB.

In subsequent experiments, *E. coli*-expressed histidine-tagged CPEB bound to Xenopus oocyte Eg2, when oocyte extract was applied to a column containing His-CPEB.

Example 5: Cancer Therapy

The blocking polypeptides are useful for inhibiting cancer growth. To evaluate the role of the blocking polypeptides in prostate cancer progression, the blocking polypeptides are administered to a transgenic adenocarcinoma mouse prostate (TRAMP) model (see, e.g., U.S. Patent No. 5,907,078). More particularly, sixty 12-week-old male TRAMP mice are

placed randomly into two groups. The animals are treated by daily oral gavage with vehicle (1% water) or blocking polypeptides for 18-weeks. Following this time period, the prostate lobes, seminal vesicles, lungs, and periaortic lymph nodes are preserved and sectioned for histological evaluation. Growth of cancerous prostate cells is assessed and compared in test and control mice.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

We claim:

1. A substantially pure polypeptide of 5 to 100 amino acids, the polypeptide comprising the amino acid sequence LDXR (SEQ ID NO:4), wherein X is selected from the group consisting of G, A, V, L, I, M, C, S or T.

2. The polypeptide of claim 1, wherein X is selected from the group consisting of A, V, L, I, S or T.

3. The polypeptide of claim 1, wherein X is A.

4. The polypeptide of claim 1, wherein the polypeptide comprises amino acid sequence LRSSRLDXRSILDSRSS (SEQ ID NO:3), or SEQ ID NO:3 with one or more conservative amino acid substitutions, provided that LDXR is preserved.

5. The polypeptide of claim 4, wherein X is A, V, L, I, S or T.

6. The polypeptide of claim 4, wherein X is A.

7. The polypeptide of claim 1, wherein the polypeptide comprises amino acid sequence RGSRLDXRPILDSRS (SEQ ID NO:6), or SEQ ID NO:6 with one or more conservative amino acid substitutions, provided that LDXR is preserved.

8. The polypeptide of claim 7, wherein X is A, V, L, I, S or T.

9. The polypeptide of claim 7, wherein X is A.

10. The polypeptide of claim 1, wherein the polypeptide is selected from the group consisting of LRSSRLDSRSILDSRSS (SEQ ID NO:3); SEQ ID NO:3 wherein the serine residue at amino acid position 8 is phosphorylated; RSSRLDARSILDSRSS (SEQ ID NO:8); RGSRLDTRPILDSR (SEQ ID NO:4); and SEQ ID NO:4 wherein the threonine residue is phosphorylated.

11. An isolated nucleic acid encoding the polypeptide of claim 1.

12. A vector comprising the nucleic acid of claim 11.

13. A cell comprising the vector of claim 11.

14. A method of inhibiting the activity of kinase Eg2, the method comprising contacting the kinase Eg2 with an effective amount of the polypeptide of claim 1.

15. A method of inhibiting the phosphorylation of CPEB in a cell, the method comprising contacting Eg2 in the cell with an effective amount of the polypeptide of claim 1.

16. The method of claim 15, wherein the method is performed in vivo.

17. The method of claim 15, wherein the method is performed in vitro.

18. A method of producing a polypeptide, the method comprising culturing a cell comprising the nucleic acid of claim 11, under conditions and for a time sufficient to enable the cell to express a polypeptide encoded by the nucleic acid, and isolating the polypeptide from the cell.

19. An isolated antibody that specifically binds to the polypeptide of claim 1.

20. The isolated antibody of claim 19, wherein X represents a phosphorylated S or T residue.

21. A method of detecting CPEB in a sample, the method comprising;

contacting the antibody of claim 19 to the sample under conditions that enable the antibody to bind to CPEB to form a CPEB-antibody complex, if present, and detecting any complexes, the presence of a complex indicating the presence of CPEB in the sample.

22. The method of claim 21, wherein the CPEB is phosphorylated.

23. A solid support comprising an antibody of claim 19 bound thereto.

24. A method of treating a cancer cell, the method comprising:

contacting the cell with an effective amount of a blocking polypeptide of claim 1; thereby treating the cancer cell.

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                *
Xenopus     RSSRLDSRSILDSR------SSSP
Mouse       RGSRLDTRPILDSR------SSSP
Zebrafish       RDPLKLALDSRMDHSSSP
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FIG. 1

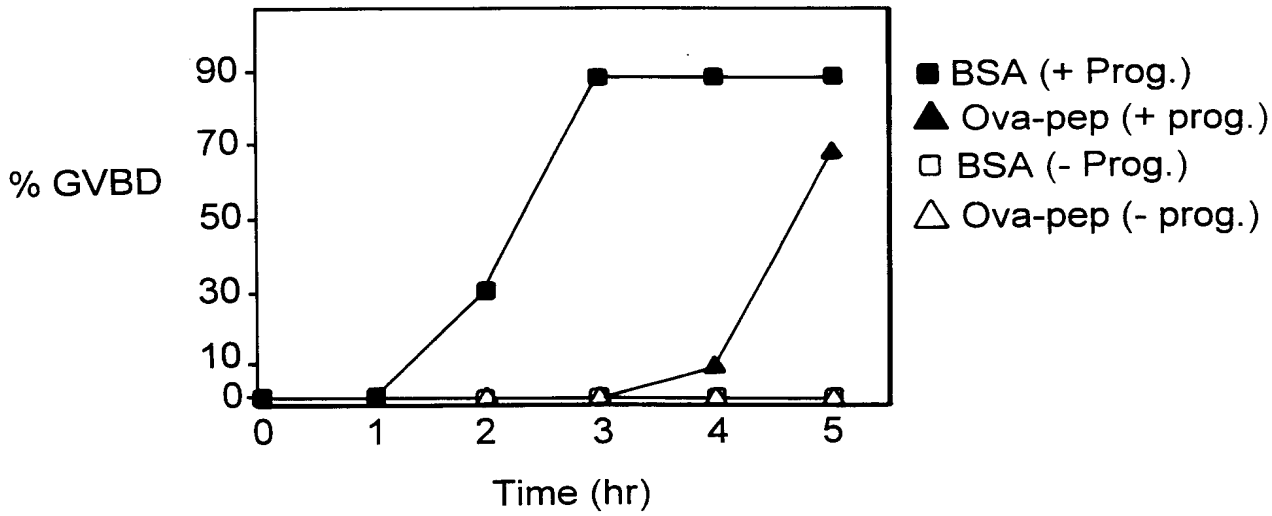
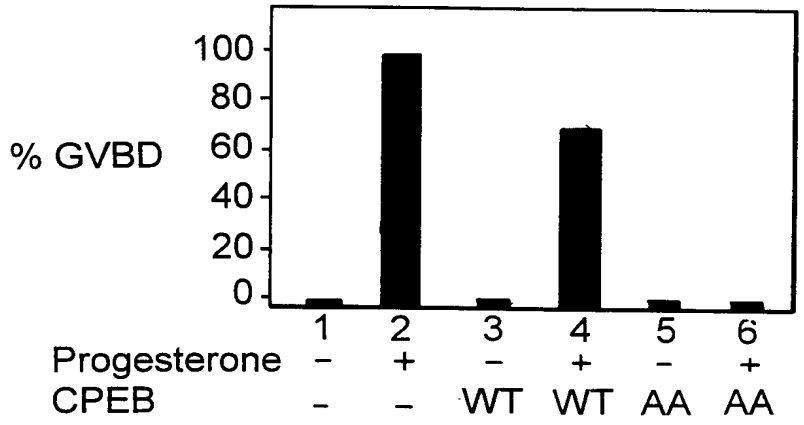


FIG. 3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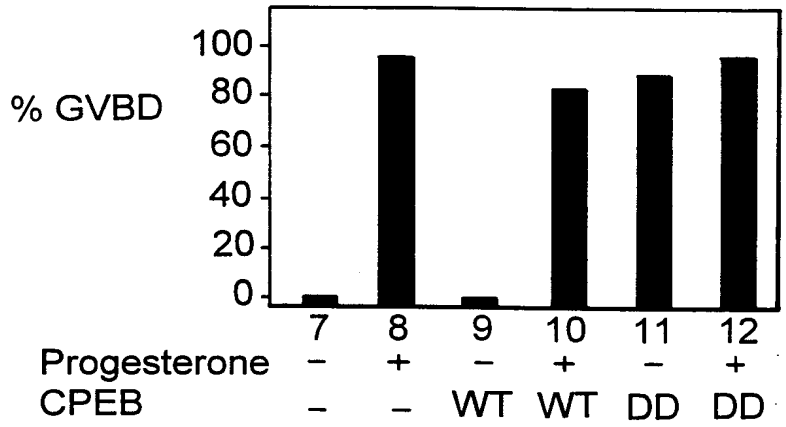
80
60
40
20
0
1 2 3 4 5 6
Progesterone – + – + – +
CPEB – – WT WT AA AA

FIG. 2A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80
60
40
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0
7 8 9 10 11 12
Progesterone – + – + – +
CPEB – – WT WT DD DD

FIG. 2B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/20254

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07K 5/00
US CL : 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/330 530/327

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, EAST/WEST, STIC SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	MENDEZ, R. et al, Nature, 16 March 2000, Vol. 404, pp. 302-307, entire text.	1-10
X	HAKE, L. et al, 1994, Cell, Vol. 79, pp. 617-627 see Figure 3A on pg. 620.	1-10
X	GEBAUER, F., 1997, EMBL, ID P70166	1,2,4,5,7,8
A	GEBAUER, F., et al, PNAS, 1996, Vol 93, pp. 14602-14607, entire document.	1-10
A	PARIS, J., et al, Genes and Development, 1991, Vol. 5, pp. 1697-1708, entire document.	1-10
A	LEGAGNEUX, V., et al, Development, 1992, Vol. 116, pg. 1193-1202, entire document.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
	09 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer GEETHA BANSAL Telephone No. 703-308-1234

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